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Nonionic surfactants increase digoxin absorption in Caco-2 and MDCKII MDR1 cells: Impact on P-glycoprotein inhibition, barrier function, and repeated cellular exposure.

Running title: Nonionic surfactants alter digoxin permeability

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Abstract

Nonionic surfactants commonly used in pharmaceutical formulations may have P-glycoprotein (P-gp) inhibiting and/or permeation enhancing effects. The present work aims to distinguish these effects and assess the degree of cellular recovery after multiple exposures to nonionic surfactants. The investigated surfactants were polysorbates (PS): PS20, PS40, PS60, PS65, PS80 and PS85; monosaccharide-based: lauroyl methyl glucamide and n-nonyl-β-D-glucopyranoside; or disaccharide-based: lauryl-β-D-maltoside and trehalose 6-laurate. Bi-directional permeability studies of digoxin and mannitol, and calcein-AM efflux assay were performed in cell cultures. Cellular recovery was evaluated by continuous measurements of transepithelial electrical resistance (TEER) in Caco-2 cell monolayers. Polysorbates with one fatty acid chain decreased the efflux of digoxin through P-gp inhibition in MDCKII MDR1 cells. Mono- and di-saccharide-based surfactants, in a dose dependent manner, enhanced digoxin absorptive permeability without decreasing the secretory permeability in Caco-2 cells, suggesting that the surfactants had a transcellular permeation enhancing effect. Caco-2 cell monolayers recovered to different degrees of 60-100% of the initial TEER values. Calcein-AM assay was found to be non-predictive to surfactants influence on digoxin permeability across cell monolayers. In conclusion, these results may assist, in a mechanism-based, selection of suitable surfactants for formulating oral dosage forms to enhance the absorption of low bioavailable P-gp substrates.

Keywords: Nonionic surfactant, P-glycoprotein, digoxin, Calcein-AM, Caco-2, MDCKII MDR1 and TEER.

1. Introduction

A pharmaceutical formulation approach is needed to enhance the intestinal absorption of drug substances with low oral bioavailability limited by P-glycoprotein (P-gp, ABCB1, MDR1) mediated efflux. Nonionic surfactants have been reported to reverse multi-drug resistance in cancer cells (Coon et al., 1991; Woodcock et al., 1990; Woodcock et al., 1992). Later studies showed that some surfactants, such as polysorbate 80, cremophor EL (Hugger et al., 2002; Rege et al., 2002), polysorbate 20, Myrj 52 and Brij 30 (Lo, 2003), could increase the permeability of P-gp substrates in cell cultures. A part of the increased permeability is related to inhibition of P-gp mediated cellular efflux. It has been suggested that the mechanism behind surfactant mediated inhibition of P-gp, may be facilitated by the hydrophobic tail of the surfactants, which partition into the membrane bilayer, while the hydrophilic moiety form hydrogen bonds with hydrogen bond donor groups in the protein (Li-Blatter et al., 2012; Li-Blatter et al., 2009; Li-Blatter and Seelig, 2010; Xu et al., 2015).

Even though most work on the ability of nonionic surfactants to inhibit P-gp has been performed *in vitro*, a few studies have investigated if surfactants administered orally to animals could increase the bioavailability of P-gp substrates, such as digoxin (Cornaire et al., 2004; Nielsen et al., 2016; Zhang et al., 2003), doxorubicin (Al-Saraf et al., 2016), and etoposide (Akhtar et al., 2017; Al-Ali et al., 2018). In wild type Sprague-Dawley rats, polysorbate 20 significantly increased the oral bioavailability of digoxin (Nielsen et al., 2016) and etoposide (Al-Ali et al., 2018). However, oral co-administration of polysorbate 20 with digoxin or etoposide in *mdr1a* deficient Sprague-Dawley rats has had no impact on the bioavailability, compared to the oral administration of these P-gp substrates without the surfactant (Al-Ali et al., 2018; Nielsen et al., 2016). This suggests that polysorbate 20 mediated P-gp inhibition is the reason for the increased bioavailability in wild type rats.

Moreover, it has been proposed that nonionic surfactants dose dependently can be used as intestinal permeation enhancers (Deli, 2009; Hochman and Artursson, 1994; Maher et al., 2016) by opening the tight junctions of epithelial cells allowing for paracellular transport enhancement (Gradauer et al., 2017; Ujhelyi et al., 2012), or through surfactant monomeric-phospholipid exchange enhancing transcellular transport (Maher et al., 2018; Maher et al., 2016), or through a combination of both mechanisms (Doo et al., 2005; Petersen et al., 2012). A successful candidate permeation enhancer should have a temporary and significant permeation enhancing effect, post exposure epithelial recovery, and a sufficient safety profile (Maher et al., 2016). It can therefore be hypothesized that a nonionic surfactant may possess a P-gp inhibition effect and/or a permeation enhancing effect, which may influence the exposure of drug substances that are P-gp substrates when co-administered with nonionic surfactants. Distinguishing these effects and understanding the underlying mechanisms are thus important and useful for selecting suitable excipients, which may improve formulating P-gp substrate drug substances in oral dosage forms.

In the present study, two sets of nonionic surfactants were selected, the first set includes a range of polysorbates that contain similar hydrophilic moiety, but different hydrophobic tails and the second set includes a range of surfactants with similar hydrophobic laurate-based tail, but different hydrophilic moieties such as mono- and di-saccharides. In addition, a monosaccharide-based nonionic surfactant with shorter alkyl chain n-nonyl-β-D-glucopyranoside was also investigated (Table 1 and Figure 1). The aim of the present work was to investigate the ability of these nonionic surfactants to modulate P-gp function, to distinguish between the P-gp modulation and/or permeation enhancing effects of the surfactants, and to evaluate TEER recovery of cell monolayers after multiple exposures to surfactants, P-gp substrate or co-solvents.

2. Material and methods

2.1 Materials

MDCKII MDR1 cells were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands) and Caco-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). Transwell^{TM} (polycarbonate membrane, 0.4 µm pore size) inserts (1.12 cm^2) and black 96-well microplates with clear bottom were from Corning Life Sciences and purchased through Sigma Aldrich (Broendby, Denmark). Calcein acetoxymethyl ester (calcein-AM), quinidine, verapamil, valspodar, zosuquidar, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and trehalose 6-laurate (TL) were at purities of ≥ 95% and from Sigma Aldrich (Broendby, Denmark). Ethanol (96%) was from VWR Chemicals (Soeborg, Denmark). Dulbecco's modified Eagle medium (DMEM), Fetal Bovine Serum (FBS), penicillin/streptomycin (100X), L-glutamine (200 mM), non-essential amino acids (1%), sodium bicarbonate solution (7.5%) and Trypsin-EDTA (10X) were suitable for cell culture, sterile-filtered and from Sigma Aldrich (Broendby, Denmark). Hanks Balanced Salt Solution (HBSS) (10X) buffer was suitable for cell culture, sterile-filtered, and purchased from Gibco through Thermo Fisher Scientific (Roskilde, Denmark). Lauryl-β-D-maltoside (LM) (purity of ≥ 99%) was from Thermo Fisher Scientific (Roskilde, Denmark). Lauroyl methyl glucamide (LMG) and n-nonyl-β-D-glucopyranoside (NG) were at purity of ≥ 97% and from Bachem (Bubendorf, Switzerland). Polysorbate surfactants (Table 1) were cell culture tested and from Sigma Aldrich (Broendby, Denmark). Polysorbates were specified according to Ph. Eur. 7.4 containing ≤ 3% water. ^{3}H-digoxin (specific activity 39.8 mCi $mmol^{-1}$), ^{14}C-mannitol (specific activity 57.2 mCi $mmol^{-1}$), Ultima Gold scintillation fluid, and scintillation vials were from Perkin Elmer (Skovlunde, Denmark). Radiochemical purity of the isotopes was greater than 97%.

2.2 Cell culture

MDCKII MDR1 cells were seeded on black 96-well microplates (clear bottom) at 1.43 10^5 cells cm^{-2} and used four days after seeding. Permeability studies were performed on MDCKII MDR1 and Caco-2 cells seeded on Transwell^{TM} inserts at 2.9 10^5 and 8.9 10^4 cells cm^{-2}, respectively. MDCKII MDR1 and Caco-2 cells were cultured as described previously for 4-5 and 12-15 days, respectively, before the permeability studies were conducted (Al-Ali et al., 2018; Nielsen et al., 2016). In short, the cells were maintained in an incubator at 37 ^oC in a humidified atmosphere supplemented with 5% CO_2. The culture medium was changed every 2–3 days and consisted of DMEM supplied with 10% FBS, penicillin/streptomycin (10,000 U ml^{-1}/10 mg ml^{-1}), L-glutamine (1%), and non-essential amino acids (1%).

2.3 Calcein-AM efflux assay

Calcein-AM efflux experiments were performed on MDCKII MDR1 cells, which were seeded on black 96-well microplates. Before the experiments, media was removed from the wells and then to each well 50 µL pre-warmed HBSS supplemented with 10 mM HEPES adjusted to pH 7.4 ± 0.05 ($HBSS^+$) was added. After a 15 min incubation period, the $HBSS^+$ buffer was removed and 50 µL of solutions containing P-gp inhibitors or nonionic surfactants dissolved in $HBSS^+$ were added to the wells. Each plate was then incubated at 37 ^oC on a shaking plate at 220 rpm for 15 minutes before calcein-AM was added to each well to a final concentration of 12.5 µM. Calcein-AM diffuses into the cell where it is either hydrolysed to in membrane-impermeable fluorescent calcein or extruded from the cell by P-gp. Inhibiting P-gp increases the amount of calcein in the cell and a larger amount is hydrolysed to calcein, thus the fluorescence increased. Cells were incubated in

absence or presence of verapamil, quinidine, valspodar and zosuquidar in a concentration range of 0.01-800, 0.04-1000, 0.01-30, 0.0001-10 µM, respectively. Polysorbates were used at a concentration range of 0.02-2000 µM for PS20, 2-1000 µM for PS40, PS60 and P80, and 2-2000 µM for PS65 and PS85. LM and TL were investigated at a concentration range of 2-2000 µM. LMG and NG were investigated at 2-1250 and 20-3200 µM, respectively. The highest concentration of each compound was added to a cell-containing well in the absence of calcein-AM to investigate if compounds were fluorescent themselves. Gain adjustment (excitation 485 nm and emission 520 nm) was obtained from the well expected to have the highest response of fluorescent calcein before the continuous measurements of fluorescence (excitation 485 nm and emission 520 nm) was initiated. The measurements were recorded for 30-60 cycles (60 sec $cycle^{-1}$) at 37 ^{o}C using a FLUOstar Omega plate-reader from BMG Labtech (Ortenberg, Germany).

2.4 Permeability studies across MDCKII MDR1 and Caco-2 cell monolayers

Absorptive (A-B) and secretory (B-A) permeabilities of ^{3}H-digoxin were investigated across Caco-2 cell monolayers in the absence or presence of increasing concentration of nonionic surfactants which were prepared in $HBSS^{+}$. In MDCKII MDR1, the permeability studies were performed in the absence or presence of 200 µM of a polysorbate or 10 µM zosuquidar which were prepared in $HBSS^{+}$. The surfactant containing solutions were only applied to the apical side of cell monolayers, while the zosuquidar solution was added to the apical and the basolateral side. LM, TL and LMG were used in a concentration range of 20-200 µM and NG at 400-1600 µM in Caco-2 cells. After the cells were removed from the incubator, the cell culture media were removed and the cells were pre-incubated in $HBSS^{+}$ on a shaking plate at (220 rpm, 37^{o}C) for 15 minutes. The media were then removed, and replaced with isotope containing solutions in the upper (500 µL) or lower chamber

(1000 µL) for absorptive or secretory permeability measurements, respectively. Donor samples (20 µL) of isotopes-containing solutions were collected at the start and end of experiments. Samples from the apical (50 µL) or basolateral (100 µL) receiver chambers were collected at 20, 40, 60, 90 and 120 minutes after addition of the isotopes to the donor side. At 120 minutes, filters with cell monolayers were washed three times using ice cold HBSS solution, then cut from the plastic support and transferred to 6 mL pony scintillation vials. Ultima Gold scintillation liquid (2 mL) was added to every sample, vortexed for one minutes and counted on a liquid scintillation counter (TriCarb 4910TR) from Perkin Elmer (MA, USA) for 10 min.

2.5 Measurement of transepithelial electrical resistance of Caco-2 and MDCKII MDR1 cell monolayers

Caco-2 and MDCKII MDR1 cell monolayers integrity were evaluated before and after each permeability experiment with nonionic surfactants by measuring the transepithelial electrical resistance (TEER). Transwell^{TM} inserts seeded with MDCKII MDR1 or Caco-2 cells were placed in 12 mm Culture Cup (ENDOHM-12) connected with EVOM2 Volt/Ohm Meter from World Precision Instruments (Sarasota, FL) and the resistance (Ω) was measured. The resistance was corrected for the polycarbonate membrane and fluid resistance, and then multiplied with the surface area of the membrane to obtain the TEER (Ω cm^2).

Continuous TEER measurements after multiple exposures to P-gp substrate digoxin, nonionic surfactants, or co-solvents were recorded every 17-20 min for approximately 27 h using cellZscope from nanoAnalytics GmbH (Munster, Germany). Transwell^{TM} insert, seeded with Caco-2 cells ,was placed in cellZscope chamber and 760 µL medium supplemented with 10 mM HEPES and adjusted to a pH of 7.40 ± 0.05 ($DMEM^+$) was applied on the apical side of cell monolayers followed by

$HBSS^+$ (1650 µL) in the basolateral side. After the baseline measurements, $HBSS^+$ were removed followed by $DMEM^+$ and the monolayers were washed two times with pre-warmed $HBSS^+$ before compounds-containing solutions prepared in $HBSS^+$ or $DMEM^+$ were applied on the apical side. $HBSS^+$ was usually applied in the basolateral side. Experiments were performed at 25°C and shaking mode of 80 rpm used. Digoxin was used at 100 µM and 25 nM with or without 500 µM PS20. PS20 was used at 500 µM with or without digoxin because this was the maximum dose of PS20 previously investigated to inhibit P-gp in cell culture studies in our laboratory (Al-Ali et al., 2018; Nielsen et al., 2016). LM, TL and LMG were investigated at 200 µM, and NG at 1600 µM. Ethanol and DMSO were investigated at 1 and 5 % (v/v). Cell monolayers were exposed to different compounds or controls for 2 h intervals at 0.5, 5.5 and 21.5 h after initiating the experiments. Cell monolayers were washed for two times with pre-warmed $HBSS^+$ after each exposure and replaced with either $HBSS^+$ after the first and third exposure or with $DMEM^+$ after the second exposure for overnight incubation.

2.6 Data analysis

Calcein fluorescence in arbitrary unit (a.u) corrected for background signal, which was the signal obtained from the cells incubated with $HBSS^+$ without calcein-AM, was plotted as a function of time and slopes (denoted m below) were obtained by linear regression. The increase in fluorescent calcein is a surrogate marker of increased P-gp inhibition (Holló et al., 1994). The slope from curves of fluorescence as a function of time was normalized to the slope of fluorescence in cells exposed to $HBSS^+$ only (referred as control). In the present work the inverse relationship of the normalized response (modified from (Caetano-Pinto et al., 2016)) was obtained as a calcein-AM efflux in % of control using equation 1:

$$\text{Calcein-AM efflux in \% of control} = \left(1 \div \left[\frac{m_{compound}}{m_{control}}\right]\right) \text{x } 100 \quad \text{Eq. 1}$$

Where $m_{control}$ and $m_{compound}$ are the slopes obtained in cell exposed to $HBSS^+$ or in the presence of a compound, respectively. The calcein-AM efflux in % of control was plotted as a function of logarithmic concentrations of compounds, and IC_{50} values were obtained by fitting the data to nonlinear regression using the three parameter equation in GraphPad Prism 7.01 (See equation 2). The bottom values were constrained to be larger than zero for PS65, LMG and NG.

$$Y = \frac{\text{Bottom} + (\text{Top-Bottom})}{(1+10^{(\text{X-Log } IC_{50})})} \quad \text{Eq. 2}$$

For transport studies, the radioactivity of samples in disintegrations per minute (dpm) were converted to equivalent mass. The accumulated mass of ^{3}H-digoxin and ^{14}C-mannitol in the receiver chambers per cell culture area (pmol cm^{-2}) was plotted as a function of time and the steady state slope was obtained as the flux (J) in pmol cm^{-2} min^{-1}. The absorptive (A-B) and secretory (B-A) apparent permeability coefficient (P_{app}) was calculated using (equation 3) derived from Fick's first law, where C_0 is the initial concentration of ^{3}H-digoxin or ^{14}C-mannitol in the donor compartment at the start of experiment.

$$P_{app} = \frac{J}{C_0} \quad \text{Eq. 3}$$

Efflux ratio (ER) was obtained using equation 4.

$$ER = \frac{P_{app\ (B-A)}}{P_{app\ (A-B)}} \quad \text{Eq. 4}$$

The recovery of ^{3}H-digoxin in percentage from permeability studies was calculated using equation 5, where dpm_s is the dpm of samples in the receiver compartment at time points 20, 40, 60 and 90 min, dpm_{s120} is the dpm of the sample at 120 min, dpm_{d120} is the dpm of the donor compartment at

120 min, dpm_m is the dpm in the polycarbonate membrane with cell monolayer, dpm_{d0} is the dpm of the donor compartment at time zero.

$$\text{Recovery (\%)} = \frac{[\left(\sum_{20}^{90} dpm_s + (dpm_{s120} \times 10)\right) + dpm_{d120} + dpm_m]}{dpm_{d0}} \times 100\% \qquad \text{Eq. 5}$$

Intracellular accumulation of digoxin in MDCKII MDR1 and Caco-2 cells at the end of the permeability studies were calculated after correction of dpm present in the extracellular space using a mannitol volume of distribution (vd_{man}) (Bravo et al., 2005). The corrected Intracellular accumulation of digoxin in pmol was calculated using equation 6, where dpm_{ds0} and v_{ds0} is the dpm and volume of the donor sample at time zero, respectively.

$$\text{Intracellular accumulation of digoxin} = \frac{dpm_m - \left(\frac{vd_{man} \times dpm_{ds0}}{v_{ds0}}\right)}{\text{Specific activity}_{digoxin}} \qquad \text{Eq. 6}$$

Chemical structures of the investigated compounds were made using ChemDraw 16.0 from Perkin Elmer Informatics (Cambridge, MA, USA).

2.7 Statistics

Data were obtained from at least three independent passages (n=3). The results are presented as mean values ± SEM and statistical analysis was performed by utilizing GraphPad Prism 7.01. One-way ANOVA test followed by Holm-Sidak's multiple comparisons test were used to compare different groups with control. P value < 0.05 were considered as statically significant from control and referred as (*).

3. Results

3.1 Nonionic surfactants alter P-glycoprotein activity in MDCKII MDR1 cells

The efflux of calcein-AM was investigated in MDCKII MDR1 cells in the presence of P-gp inhibitors and nonionic surfactants. The ability of these compounds to increase intracellular fluorescence, hence inhibit P-gp activity, was investigated as a function of concentration (see supplementary figure S1), and based on this, IC_{50} values were estimated (Table 2). The positive controls zosuquidar, valspodar, verapamil and quinidine showed IC_{50} values in the range 0.1-3.8 µM. The investigated nonionic surfactants showed highly different abilities to inhibit P-gp, with PS20 having an IC_{50} value of 11 µM. The other investigated polysorbates with inhibitory activities were PS40, PS60 and PS80; whereas PS65 showed weak ability to inhibit P-gp, and PS85 did not inhibit P-gp at the concentrations investigated. For nonionic surfactants with laurate hydrophobic tail and different hydrophilic moieties, the affinities were lower with IC_{50} values of 42- 88 µM for LM, TL and LMG, while NG showed a poor ability to increase intracellular calcein fluorescence, indicating a limited P-gp affinity.

3.2 Nonionic surfactants alter digoxin permeability across MDCKII MDR1 and Caco-2 cell monolayers

The ability of surfactants to increase the absorptive transport of digoxin was measured in MDCKII MDR1 and Caco-2 cells. Digoxin showed polarized secretory transport with efflux ratios of 19 (Fig. 2A and 2B) and 28 (Fig. 3A and 3B) in MDCKII MDR1 and Caco-2 cells, respectively. In MDCKII MDR1 cells, PS20 and zosuquidar were able to decrease the efflux ratio of digoxin to unity, which was caused by a significant increase in the absorptive permeability and a significant decrease in the secretory permeability of digoxin (Fig. 2A and 2B). Digoxin secretory permeability significantly decreased in the presence of 200 µM of PS60, PS80 and PS85 (Fig. 2B). It seems,

however, that PS65 did not have an impact on the absorptive or the secretory permeability of digoxin. To assess permeability enhancing effects related to paracellular pathway, mannitol permeability was evaluated, and in the presence of polysorbates or zosuquidar no increased permeability was seen (Table 3). Furthermore, TEER values after the 2 h incubation with polysorbates tended to increase rather than decrease (Table 3).

In Caco-2 cells, increasing concentrations of LM, TL, LMG and NG, on the apical side only, increased the absorptive digoxin permeability significantly for 150 and 200 µM LM, 50-200 µM TL, 100-200 µM LMG and 600-1600 µM NG (Figure 3A). In contrast to what was observed for PS20, secretory digoxin permeability was not reduced in the presence of apical concentrations of LM, TL, LMG and NG (Fig. 3B). Effects on the barrier function of the cell monolayers were observed with significantly reduced TEER values for 150-200 µM LM and TL, and a significant increase in mannitol permeability (A-B and B-A) for 200 µM LM and 150-200 µM TL (Table 3). With high concentrations of LMG and NG, TEER values were significantly decreased, but mannitol permeabilities (A-B and B-A) were not enhanced; however, digoxin absorptive permeability increased significantly. The maximal enhancement in digoxin absorptive permeability was observed with 200 µM LM or TL, and the enhanced absorptive permeability with the former surfactant was significantly higher than the enhancement with 200 µM LMG or 1600 µM NG.

3.3 Intracellular accumulation of digoxin in MDCKII MDR1 and Caco-2 cells

Intracellular accumulation of digoxin was estimated after 120 min of absorptive and secretory permeability studies (Fig. S2 and Fig. S3). In the absence and presence of surfactants or zosuquidar, the intracellular accumulation of digoxin from the basolateral side was higher than from the apical side. In the absence of nonionic surfactants (control), the intracellular accumulation of digoxin from

the apical side was low at 2.5 ± 0.4 and 2.9 ± 1 fmol in MDCKII MDR1 and Caco-2 cells, respectively. In MDCKII MDR1 cells, the intracellular accumulation of digoxin from the apical side after absorptive permeability studies increased significantly compared to control in the presence of zosuquidar, PS20, PS60 and PS80. Presence of PS65 and PS85 on the apical side of monolayers did not increase the intracellular accumulation of digoxin from the apical side when compared to the control (Fig. S2A). The presence of different polysorbates on the apical side did not increase the intracellular accumulation of digoxin from the basolateral side compared to control (Fig. S2B).

In Caco-2 cells, the intracellular accumulation of digoxin from the apical side, at the end of absorptive permeability studies was concentration dependently increased in the presence of different surfactants, and it was statistically significant at 100-200 µM LM, 50-200µM TL and LMG, 800-1600 µM NG, and 20-500 µM PS20, when compared to the control (Fig. S3A). The intracellular accumulation of digoxin from the basolateral side increased significantly in the presence of 200 µM LMG, 1200-1600 µM NG, and 20-500 µM PS20 (Fig. S3B).

3.4 Continuous measurements of transepithelial electrical resistance of Caco-2 cell monolayers after multiple exposures to nonionic surfactants

The ability of Caco-2 cells to recover after multiple exposures to nonionic surfactants, P-gp substrate and co-solvents was investigated by continuous measurements of TEER values every 17-20 min. The multiple exposure periods and the recovery periods in $HBSS^+$ or $DMEM^+$ are shown as mean values in percentage of the baseline TEER measurements in (Fig. 4), and as mean values with standard error of the mean, see Supplementary data, Fig. S4. Cell monolayers treated with $HBSS^+$ for the indicated exposure intervals and incubated with $DMEM^+$ overnight (referred to as control)

completely recovered their TEER values; however, overnight incubation in only $HBSS^+$ did not result in a complete recovery (Fig. 4A).

From figure 4A, during the first exposure, it can be seen that the maximal reduced TEER values were with monolayers treated with LM. When LM was removed and replaced with $HBSS^+$, TEER values stayed at the same range, at approximately 65%, before the TEER values was further reduced with the second exposure with LM. When LM was removed and the monolayers incubated overnight with $DMEM^+$, TEER values increased to mean values of approximately 85%. In the presence of TL, the TEER values reduced to approximately 80% during the first exposure and further reduced to around 40% during the second exposure. The monolayers were completely recovered after the surfactant was removed and monolayers incubated overnight with $DMEM^+$. LMG decreased the TEER values similar to the effect of TL during the first and second exposure. After LMG removal and overnight incubation with $DMEM^+$, the TEER values continued to reduce reaching mean values of a quarter of the baseline at nine h after the initiation of experiments. The TEER values then increased reaching around 70% of the baseline TEER measurements after 21 hours. NG did not reduce the TEER values of the monolayers during the first exposure, however, the TEER reduced upon NG removal and replacement with $HBSS^+$. Further reduction in TEER values was noticed during the second exposure with this surfactant. The TEER values further dropped during the overnight incubation with $DMEM^+$, similar to LMG, before the monolayer started to recover reaching a mean of approximately 80% of the baseline TEER values.

For monolayers treated with PS20 with or without digoxin (Fig. 4A and 4B), there was no reduction in TEER values during the first exposure; however, the decrease started afterwards during the incubation with $HBSS^+$ and the second exposure. After overnight incubation with $DMEM^+$, the monolayers recovered to approximately 60% of the baseline TEER values. The minimal degree of

recovery of Caco-2 cell monolayers exposed to different nonionic surfactants was observed with the monolayers exposed to multiple doses of PS20.

The TEER values slightly decreased in the presence of low and high dose of digoxin during the first exposure, see Fig. 4B. When different concentration of digoxin were removed and replaced with $HBSS^+$, TEER values further decreased to approximately 75% of the baseline TEER values. The second exposure with digoxin did not change the TEER values. After removal of different doses of digoxin and replacement with $DMEM^+$, Caco-2 cell monolayers recovered as the control. The data suggested that the cell monolayers were able to recover after multiple exposures to different doses of digoxin; whereas the presence of PS20 together with these compounds resulted in a partial recovery of the monolayers mainly due to the effect of PS20.

1 or 5% ethanol slightly decreased the TEER values during the first exposure (Fig. 4C). Removal of ethanol and incubating the monolayers with $HBSS^+$ further reduced the TEER. The second exposure with different concentrations of ethanol reduced the TEER values further, however, the reduction of TEER values during the previous exposures did not result in TEER values less than the TEER values of the control. Removal of ethanol and overnight incubation with $DMEM^+$ resulted in a complete recovery of the monolayers.

From figure 4C, it can be seen that 1% DMSO reduced the TEER values in a range similar to the control, during the first and second exposures. When exposed to 1% DMSO, the monolayers recovered to approximately 75% of the baseline TEER measurements in $DMEM^+$. 5% DMSO reduced the TEER values to approximately 50% in the first exposure, whereas no TEER could be measured after the second exposure. The monolayers were not able to recover when 5% DMSO was removed and the cells were incubated overnight with $DMEM^+$. The third exposure with different nonionic surfactants caused either no change or a slight decrease in TEER values except with LM

and TL where the TEER values dropped sharply. The monolayers were not able to recover in $HBSS^{+}$ at the end of experiments.

4. Discussion

The main finding of the present study was that nonionic surfactants of the polysorbate group with one fatty acid residue were better than other surfactants, such as polysorbate 65 and polysorbate 85 which have three fatty acid residues, at inhibiting P-gp transport activity, and thus increasing the absorptive permeability of digoxin. Mono- and di-saccharide-based surfactants enhanced the absorptive permeability of digoxin in a non-P-glycoprotein-dependent manner. It was also evident that the investigated surfactants altered the barrier properties, an effect that was reversible to various degrees after removing the surfactant. Another important finding was that the estimated ability of a surfactant to inhibit P-gp dependent efflux of calcein-AM was not predictive for its ability to alter P-gp substrate efflux ratio in Caco-2 and MDCKII MDR1 cell monolayers. Collectively, these findings may be useful when defining the composition of future enabling formulations in e.g. lipid based formulations.

4.1 Nonionic surfactants were either P-glycoprotein inhibitors or permeation enhancers in a non-P-glycoprotein dependent manner

Digoxin and mannitol permeability studies with mono- and di-saccharide-based surfactants were conducted using Caco-2 cells, as this in vitro model is recommended by FDA for P-gp inhibitor investigations (FDA, 2017), and has been used for investigating intestinal permeation enhancers

(Maher et al., 2016; McCartney et al., 2016). Polysorbates influencing digoxin permeability and P-gp inhibition have previously been investigated in Caco-2 cells (Nielsen et al., 2016). To investigate differences in P-gp inhibitory effects of the polysorbates, MDCKII MDR1 cells were used since the culture time is shorter and the cells overexpress the transporter in question.

In accordance with previous studies, zosuquidar (Mease et al., 2012) and PS20 (Nielsen et al., 2016) increased absorptive and decreased secretory digoxin permeability across MDCKII MDR1 cell monolayers. In Caco-2 cells, PS20, PS60 and PS80 have previously been shown to modulate the absorptive and secretory permeability of digoxin (Nielsen et al., 2016). In MDCKII MDR1 cells, the modulating effect of polysorbates was, however, mainly observed as a decreased secretory permeability of digoxin, with no effect of PS60 and PS80 on absorptive permeability. A possible explanation for the lack of increased absorptive permeability when the secretory permeability of digoxin was decreased could be based on differences in digoxin permeability across the apical and basolateral membranes. When zosuquidar was provided to both the apical and basolateral compartments giving complete inhibition of P-gp, the intracellular accumulation of digoxin was approximately ten times higher when the cells were exposed to digoxin from the basolateral side than from the apical side. This may suggest that the permeability of digoxin was higher across the basolateral membrane than across the apical membrane under P-gp saturated conditions. Therefore, inhibition of the apical efflux step of digoxin was facilitated by P-gp and the secretory transport was therefore inhibited more easily by the surfactants than the absorptive transport. Previous studies showed that several P-gp inhibitors such as GW918 (Troutman and Thakker, 2003) and verapamil (Shen et al., 2006), surfactants such as Brij 72 (Zhao et al., 2016), polyethylene glycol (PEG) mono -laurate, -oleate, and -stearate, and co-surfactants such as PEG 400 and PEG 20000 (Shen et al., 2006) decreased the secretory permeability but did not affect the absorptive permeability of the P-gp substrate rhodamine 123 in vitro. Similar effects on the secretory and absorptive permeability of

colchicine were reported with the P-gp inhibitor GF120918 in MDCKII MDR1 cells (Rautio et al., 2006). Moreover, PS65 and PS85 had lower to non-modulating effect on digoxin permeability and in the presence of these surfactants, no enhanced intracellular accumulation of digoxin was observed. It could be speculated that the presence of multiple alkyl chains in PS65 and PS85 prevented the hydrophilic moiety from getting access to the binding site of P-gp.

Previous research showed that polysorbates enhance the transcellular permeation of different compounds, such as p-aminobenzoic acid across colonic rat tissue using 0.8-8 mM of PS20, PS40, PS60 or PS80 (Sakai et al., 1986), metformin across Caco-2 cells using 8-40 mM PS20 (Dimitrijevic et al., 2000), and sulfanilic acid across rectal rat tissue using 40 mM PS20 (Nakanishi et al., 1983). It is worth noticing that high doses (0.8-40 mM) of polysorbates were required to enhance the transcellular permeation of these hydrophilic compounds, while lower dose (200 µM) of the same surfactants in the present study were needed to enhance the permeability of the lipophilic P-gp substrate digoxin in MDCKII MDR1 cells.

In Caco-2 cells, disaccharide-based surfactants LM and TL increased absorptive digoxin transport in a concentration dependent manner, but since the secretory transport was not decreased this may have been a permeability enhancing effect unrelated to inhibition of P-gp mediated efflux. Based upon evaluation of mannitol permeability, the results suggested that high doses of disaccharide-based surfactants enhanced mannitol paracellular permeability, which was most likely facilitated through tight junctions opening. Similar effects have previously been reported when LM has been used in Caco-2 (Petersen et al., 2012), in T-84, and HT-29 Cl.19A cells (Eley and Triumalashetty, 2001). In the present study, because LM and TL surfactants at high doses (150-200 µM) had an impact on the barrier function, it was not clear whether digoxin was transported by the transcellular route only. However, it was noticed that LM and TL increased the intracellular accumulation of digoxin from the apical side in a dose dependent manner. This suggested that the permeation

enhancing effect of these surfactants on digoxin permeability was mainly through transcellular route, rather than paracellular route.

LMG and NG surfactants dose dependently enhanced the absorptive permeability of digoxin across Caco-2 cells, but did not decrease the secretory permeability. Consequently these surfactants did not seem to inhibit P-gp-mediated efflux of digoxin. The surfactants did, however, enhance the intracellular accumulation of digoxin indicating the permeation enhancing effect of these surfactants on digoxin permeability was through the transcellular route. In addition, the monosaccharide-based surfactants did not enhance mannitol permeability across Caco-2 cell monolayers indicating that theses surfactants were unable to sufficiently open the tight junctions to mediate the paracellular permeability of mannitol. In agreement with these observations, a previous study has also shown that high dose of NG (160 mM), 100 times higher than the dose of NG used here, did not significantly enhance the transmucosal absorption of insulin after buccal administration in rats (Aungst, 1994). Collectively, it appears that polysorbates with only one alkyl chain inhibited P-gp activity and decrease the efflux of digoxin, and thereby improved permeability, while mono- and di-saccharide-based surfactants enhanced the digoxin permeation transcellularly, which was unrelated to P-gp inhibition.

4.2 Contribution of surfactants hydrophilic and hydrophobic moieties to modulate calcein-AM efflux

The IC_{50} values of P-gp inhibitors were estimated using the calcein AM efflux assay and the results were comparable to previous results for: 0.1 µM for zosuquidar in MDCKII MDR1 cells (Glavinas et al., 2011), 1.2 µM for valspodar in CEM/VLB_{100} human MDR1 cells (Wigler, 1999), 0.8 ± 0.1 µM in MDCKII MDR1 cells (Caetano-Pinto et al., 2016), 2.61 µM for verapamil, and 4.39 µM for

quinidine in K562-MDR cells (von Richter et al., 2009). IC_{50} values were determined for LM and NG, which were comparable to the results obtained measuring P-gp ATPase activity in mouse embryo fibroblasts transfected with *MDR1*(NIH-MDR1-G185) of 25 and 792 µM, respectively (Li-Blatter et al., 2012). Among the investigated surfactants, PS20 was the most potent surfactant for inhibiting P-gp activity. The hydrophilic polyoxyethylene moiety in PS20 seems to contribute to a larger inhibitory impact on the calcein-AM efflux than the disaccharide moiety in LM and TL, or the monosaccharide moiety in LMG, when retaining the laurate side chain in these surfactants. This could be related to a higher affinity of PS20 to P-gp, as PS20 contains a larger number of hydrogen bond accepters than LM, TL and LMG (Li-Blatter et al., 2012; Li-Blatter et al., 2009). In addition, disaccharide-based surfactants were shown to inhibit P-gp ATPase activity more than monosaccharide-based (Li-Blatter et al., 2012). This could be explained by the presence of two sugar moieties in the disaccharide-based surfactants having twice the number of hydrogen bond acceptors than the monosaccharide-based, and thus increased affinity to P-gp (Li-Blatter et al., 2012).

The hydrophilic moiety in the polysorbates is identical, but increased mono-alkyl side chain from PS40, PS60 to PS80, altered the IC_{50} values, while the presence of multiple alkyl chains in PS65 and PS85 nearly abolished the affinity for P-gp. Furthermore, monosaccharide-based surfactants, with short alkyl chain such as NG showed lower ability to decrease P-gp activity compared to monosaccharide-based surfactants with longer side chain such as the laurate moiety in LMG. In addition to the effect of the length of alkyl side chain, NG has six hydrogen bond acceptor groups, while LMG has seven, thus less affinity of NG to P-gp compared to LMG. Moreover, the cyclic pyranose in NG seems more rigid compared to the open chain form, glucamide, in LMG (see Figure 1), thus the ability of NG to access the binding sites in P-gp may be restricted. Either extending or shortening the alkyl side chain compared to laurate (12 carbon atoms) seemed to decrease the

surfactants abilities to reduce the P-gp activity. Importantly, it appeared that both the hydrophobic and the hydrophilic moieties contributed to the surfactant mediated P-gp inhibition effect which could be linked to the proposed hydrogen-bond driven mechanism of inhibition of P-gp by surfactants (Li-Blatter et al., 2012; Xu et al., 2015).

At or above the CMC of nonionic surfactants, the calcein-AM efflux continued to decrease. This was in contrast to previous studies where a reduction in P-gp inhibition was reported at or above CMC (Batrakova et al., 2004; Shono et al., 2004; Zhu et al., 2009). Interestingly, the estimated IC_{50} values of laurate containing surfactants were smaller than their CMC, while for PS40, PS60, and PS80, they were higher than their CMC, indicating that laurate containing surfactants may be effective P-gp modulator at non-micellar concentrations.

In summary, since the functional bi-directional permeability studies showed that mono- and disaccharide-based surfactants were unable to decrease the secretory permeability of digoxin, but in the calcein-AM efflux assay showed that they decreased the P-gp activity, it seems like the calcein-AM efflux assay was a poor method for prediction of P-gp inhibition mediated by these surfactants in cell culture studies.

4.3 Caco-2 cell monolayers recovered after multiple exposures to surfactants

As discussed above some of the investigated nonionic surfactants increased digoxin and mannitol transport. For further use of the surfactants, reversibility of damage to tissue barrier function and cellular toxicity is required. Recovery of cell monolayers after multiple exposures to absorption enhancers was therefore studied, as these are usually studied to distinguish whether the permeation enhancing effect is related to reversible tight junctions modulation or irreversible cellular damage due to solubilization of the plasma membrane (Eley and Triumalashetty, 2001; Moghimipour et al.,

2016; Petersen et al., 2012; Ujhelyi et al., 2012). The Caco-2 cell monolayers exposed to multiple doses of ethanol completely recovered in media, in agreement with previous research where Caco-2 cells were exposed to a single dose of 7.5% ethanol for one hour followed by 3 h incubation in Krebs buffer solution for recovery (Ma et al., 1999), or to 10% ethanol for two hours followed by 24 h recovery period in media (Catalioto et al., 2009). Likewise, a low concentration of DMSO (1%) had limited effect on Caco-2 cells recovery, while a high concentration of DMSO (5%) showed no sign of cellular recovery after overnight incubation with culture medium. Similarly, it has been reported that the TEER values of Caco-2 cells reduced after two hours exposure to 5% DMSO (Krishna et al., 2001), in a similar range as the first exposure used in the present study. In contrast to this, Yamashita and co-workers only reported a slight drop in TEER values after one hour of incubation with 5% DMSO (Yamashita et al., 2000).

During the first exposure period, TEER values did not reduce after exposure to 500 µM PS20, which was comparable to a limited effect on TEER values measured after 2 h of the initiation of permeability studies in Caco-2 cell monolayers exposed to 200 µM (Al-Saraf et al., 2016; Lo, 2003) or 500 µM PS20 (Al-Ali et al., 2018; Nielsen et al., 2016). Although the cell monolayers were not completely recovered after PS20 removal, recovery was achieved after the monolayers were exposed to other surfactants. This difference in recovery may in part be related to the high dose of PS20 (500 µM, 10 times CMC) used, when compared to LM and TL doses at 200 µM (at approximately CMC), or to 200 µM LMG and 1600 µM NG (doses < CMC), see Table 1. Importantly, LM, TL, and LMG led to a rapid decrease in TEER values, NG however did not, followed by a short time epithelial recovery, the former in agreement with previous studies (Deshmukh et al., 2010; Eley and Triumalashetty, 2001). The data suggested that LM, TL and LMG could be well tolerated surfactants for inclusion as absorption enhancers in pharmaceutical formulations.

From the data presented here, it is recommended to include polysorbates with a single alkyl chain in oral formulations designed to enhance the oral absorption of P-gp substrates. Previous studies showed that 5% (v/v) polysorbate 20 enhanced the oral absorption and bioavailability of the P-gp substrate etoposide in wild type Sprague Dawley rats (Al-Ali et al., 2018), while higher concentration of the surfactant (10-25% v/v) was needed to enhance the oral bioavailability of digoxin in rats (Nielsen et al., 2016). Further preclinical studies are therefore still needed to evaluate the effective concentrations of these surfactants required to enhance the oral absorption and bioavailability of P-gp and non-P-gp substrates. In human, it is recommended that the surfactant should be used within the Acceptable Daily Intake (ADI) such as for polysorbates is 25 mg kg^{-1} day^{-1} (Food Additives, 2015).

5. Conclusion

Polysorbates with one alkyl chain decreased digoxin efflux ratio in cell cultures, which seemed related to P-glycoprotein inhibition effect. Mono- and di-saccharide-based surfactants enhanced digoxin absorptive permeability through transcellular permeation enhancing effect. Calcein-AM efflux assay was non-predictive for the impact of surfactants on P-gp activity to modulate digoxin permeability across cellular membranes. The effect of surfactants on Caco-2 cell monolayers seemed temporary, and the monolayers recovered to different degrees when the surfactant was removed and incubated overnight with media. The present work was, therefore, able to distinguish the nonionic surfactants that possess P-glycoprotein inhibitory properties, the ones with permeation enhancing effect, and the surfactants with reversible impact on cellular membranes. Knowing these characteristics may assist when selecting suitable surfactants for pharmaceutical formulations to enhance the oral absorption of low bioavailable P-gp substrate drug substances *in vivo*.

Declaration of interest

The authors do not have any conflict of interest to report.

Author contribution

Conception and design of the study: AAAA, BS, RH and CUN. Acquisition of data: AAAA and CUN. Analysis and interpretation of data: AAAA, BS, RH and CUN. Drafting the article: AAAA and CUN. Critical revising and final approval of the version submitted: AAAA, BS, RH and CUN.

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Figures Legends

Figure 1. Chemical structures of the investigated: A) P-glycoprotein inhibitors, and B) Nonionic surfactants.

Figure 2. Apparent permeability coefficients (P_{app}) of ^{3}H-digoxin across MDCKII MDR1 cell monolayers. (A) Digoxin absorptive P_{app} (apical to basolateral, (A-B)), and (B) Digoxin secretory P_{app} (basolateral to apical, (B-A)). P_{app} was investigated in the absence of surfactant or zosuquidar (referred to as control), where cell monolayers exposed to digoxin in $HBSS^{+}$ consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05, in the presence of 10 µM zosuquidar in both sides of the cell monolayers, or 200 µM of a nonionic surfactant on the apical side only. Nonionic surfactants were polysorbate 20 (PS20), polysorbate 60 (PS60), polysorbate 65 (PS65), polysorbate 80 (PS80) and polysorbate 85 (PS85). Experiments were performed at 37 ^{o}C using ^{3}H-digoxin (1 µCi ml^{-1}, 25.13 nM) containing solutions at pH 7.40 ± 0.05. P_{app} is shown as means ± SEM from 3-9 independent cell passages. Statistical analysis was performed using One-way ANOVA followed by Holm-Sidak's multiple comparisons test. (*) when $p < 0.05$.

Figure 3. Apparent permeability coefficients (P_{app}) of ^{3}H-digoxin across Caco-2 cell monolayers. (A) Digoxin absorptive P_{app} (apical to basolateral, (A-B)), and (B) Digoxin secretory P_{app} (basolateral to apical, (B-A)). P_{app} was investigated in the absence of surfactant (referred to as control), where cell monolayers exposed to digoxin in $HBSS^{+}$ consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05, or in the presence of increasing concentrations of nonionic surfactants: Lauryl-β-D-maltoside (LM), trehalose 6-laurate (TL), lauroyl methyl glucamide (LMG), or n-nonyl-β-D-glucopyranoside (NG), on the apical side only. Experiments were performed at 37^{o}C using ^{3}H-digoxin (1 µCi ml^{-1}, 25.13 nM) containing solutions

at pH 7.40 ± 0.05. P_{app} is shown as means ± SEM from 3-18 independent cell passages. Statistical analysis was performed using One-way ANOVA followed by Holm-Sidak's multiple comparisons test. (*) when $p < 0.05$.

Figure 4. Continuous measurement of transepithelial electrical resistance (TEER) of Caco-2 cell monolayers with multiple exposures to: A) Control, where $HBSS^+$ consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05, was used in every exposure followed by overnight incubation (7.5-21.5 h) with $DMEM^+$ consisted of medium which was supplemented with 10 mM HEPES and adjusted to pH 7.4 ± 0.05; Buffer, where $HBSS^+$ was used during the whole experiments; Nonionic surfactants prepared in $HBSS^+$ solutions: 500 µM polysorbate 20 (PS20), 200 µM of lauryl-β-D-maltoside (LM), trehalose 6-laurate (TL), lauroyl methyl glucamide (LMG), or 1600 µM n-nonyl-β-D-glucopyranoside (NG); B) P-glycoprotein substrate digoxin in $HBSS^+$ or 500 µM PS20 solution; C) Co-solvents: Ethanol or DMSO which was prepared in $HBSS^+$. Experiments were performed at 25 ^oC and shaking mode of 80 rpm. The TEER values were recorded every 17-20 min, over 3-5 independent passages, and presented as means in percentage of the baseline TEER values. Grey backgrounds when monolayers were exposed to compounds. White backgrounds when cell monolayers exposed for (2.5-5.5 h) and (23.5-27 h) to $HBSS^+$, and for (7.5-21.5 h) to $DMEM^+$, except for Buffer condition, $HBSS^+$ was used during the whole experiments.

Figure S1. Concentration response curve of calcein-AM efflux in percentage of control in MDCKII MDR1 cells. Control was when the cells treated with $HBSS^+$ consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05. The calcein-AM efflux

investigated in the presence of: A) P-glycoprotein inhibitors: zosuquidar, valspodar, quinidine, or verapamil; B) Polysorbates: polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60), polysorbate 65 (PS65), polysorbate 80 (PS80), or polysorbate 85 (PS85); C) Lauryl-β-D-maltoside (LM), trehalose 6-laurate (TL), lauroyl methyl glucamide (LMG), or n-nonyl-β-D-glucopyranoside (NG). All the compounds were prepared in $HBSS^+$ solution. Data are displayed as means ± SEM of 4-17 independent passages, 1-3 replicates per experiment.

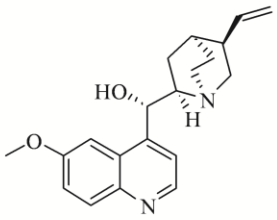
Figure S2. Intracellular accumulation of ^{3}H-digoxin (pmol) in MDCKII MDR1 cells at the end of: (A) absorptive (apical to basolateral, (A-B)), and (B) secretory (basolateral to apical, (B-A)) permeability studies. Permeability was investigated in the absence of surfactant or zosuquidar (referred to as control), where cell monolayers exposed to digoxin in $HBSS^+$ consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05, in the presence of 10 µM zosuquidar (both sides), or 200 µM of nonionic surfactant on the apical side only. Nonionic surfactants were polysorbate 20 (PS20), polysorbate 60 (PS60), polysorbate 65 (PS65), polysorbate 80 (PS80) or polysorbate 85 (PS85). Experiments were performed at 37 ^{o}C using ^{3}H-digoxin (1 µCi ml^{-1}, 25.13 nM) containing solutions at pH 7.40 ± 0.05. The intracellular accumulation is shown as means ± SEM from 3-9 independent cell passages. Statistical analysis was performed using One-way ANOVA followed by Holm-Sidak's multiple comparisons test. (*) when $p < 0.05$.

Figure S3. Intracellular accumulation of ^{3}H-digoxin (pmol) in Caco-2 cells at the end of: (A) absorptive (apical to basolateral, (A-B)), and (B) secretory (basolateral to apical, (B-A)) permeability studies. Permeability was investigated in the absence of surfactant (referred to as control), where cell monolayers exposed to digoxin in $HBSS^+$ consisted of HBSS solution,

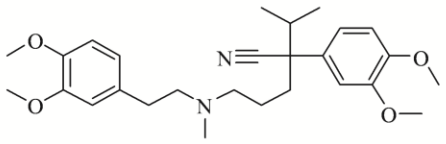
supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05, or in the presence of increasing concentrations of nonionic surfactants on the apical side only: Lauryl-β-D-maltoside (LM), Trehalose 6-laurate (TL), Lauroyl methyl glucamide (LMG), n-nonyl-β-D-glucopyranoside (NG), or polysorbate 20 (PS20). Experiments were performed at 37 ^{o}C using ^{3}H-digoxin (1 µCi ml^{-1}, 25.13 nM) containing solutions at pH 7.40 ± 0.05. The intracellular accumulation is shown as means ± SEM from 3-18 independent cell passages. Statistical analysis was performed using One-way ANOVA followed by Holm-Sidak's multiple comparisons test. (*) when $p < 0.05$.

Figure S4. Continuous measurement of transepithelial electrical resistance (TEER) of Caco-2 cell monolayers with multiple exposures to: A) Control, where HBSS^{+} consisted of HBSS solution, supplemented with 10 mM HEPES, and adjusted to pH 7.4 ± 0.05 was used in every exposure followed by overnight incubation (7.5-21.5 h) with DMEM^{+} consisted of medium which was supplemented with 10 mM HEPES and adjusted to pH 7.4 ± 0.05; Buffer, where HBSS^{+} was used during the whole experiments; Nonionic surfactants prepared in HBSS^{+} solutions: 500 µM polysorbate 20 (PS20), 200 µM of lauryl-β-D-maltoside (LM), trehalose 6-laurate (TL), lauroyl methyl glucamide (LMG), or 1600 µM n-nonyl-β-D-glucopyranoside (NG); B) P-glycoprotein substrate digoxin in HBSS^{+} or in 500 µM PS20 solution; C) Co-solvents: Ethanol or DMSO which was prepared in HBSS^{+}. Experiments were performed at 25 ^{o}C and shaking mode of 80 rpm. The TEER were recorded every 17-20 min, over 3-5 independent passages and presented as means (in percentage of the baseline TEER values) ± SEM. Grey backgrounds, when monolayers were exposed to compounds. White backgrounds, when cell monolayers exposed for (2.5-5.5 h) and (23.5-27 h) to HBSS^{+}, and for (7.5-21.5 h) to DMEM^{+}, except for Buffer condition, HBSS^{+} was used during the whole experiments.

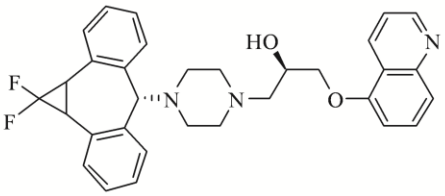
A) P-glycoprotein inhibitors



Quinidine



Verapamil



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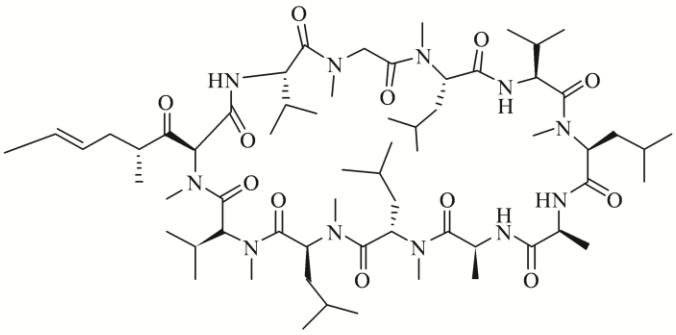
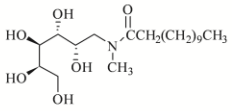
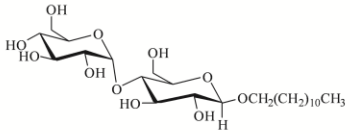


Valspodar

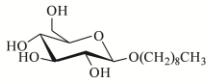
B) Nonionic surfactants



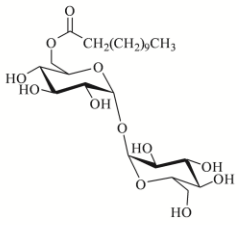
Lauroyl methyl glucamide



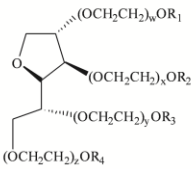
Lauryl-ß-D-maltoside



n-nonyl-ß-D-glucopyranoside

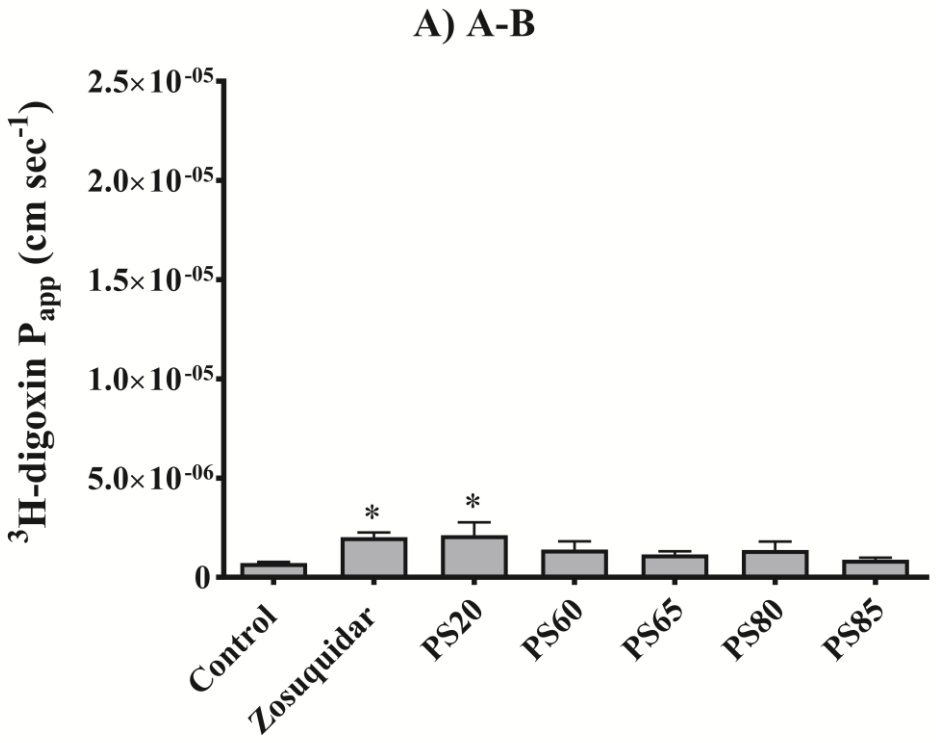


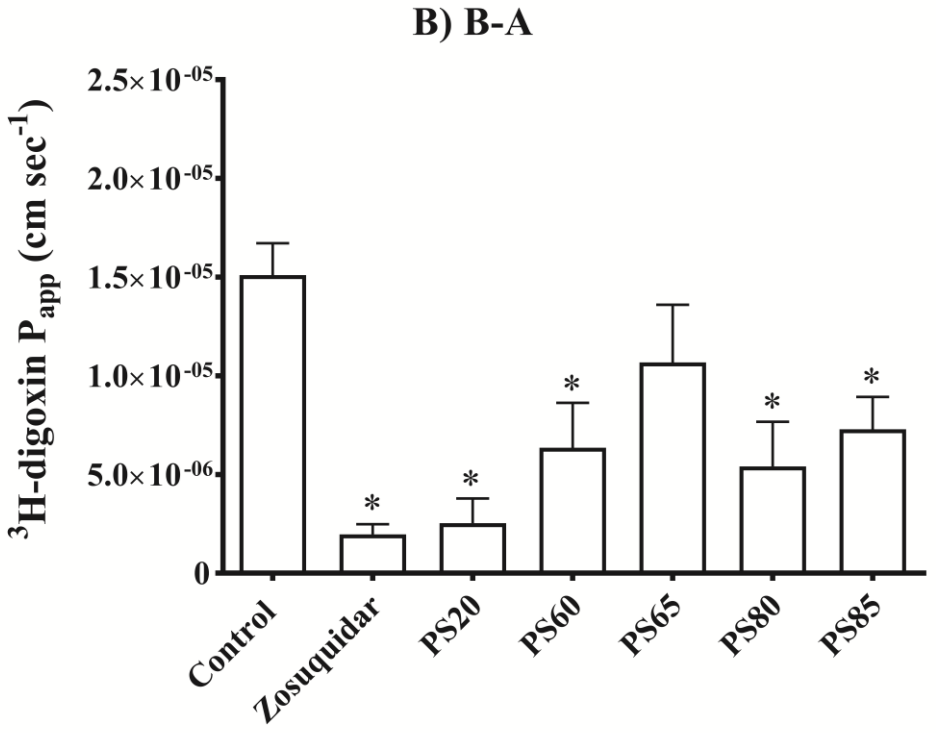
Trehalose 6-laurate

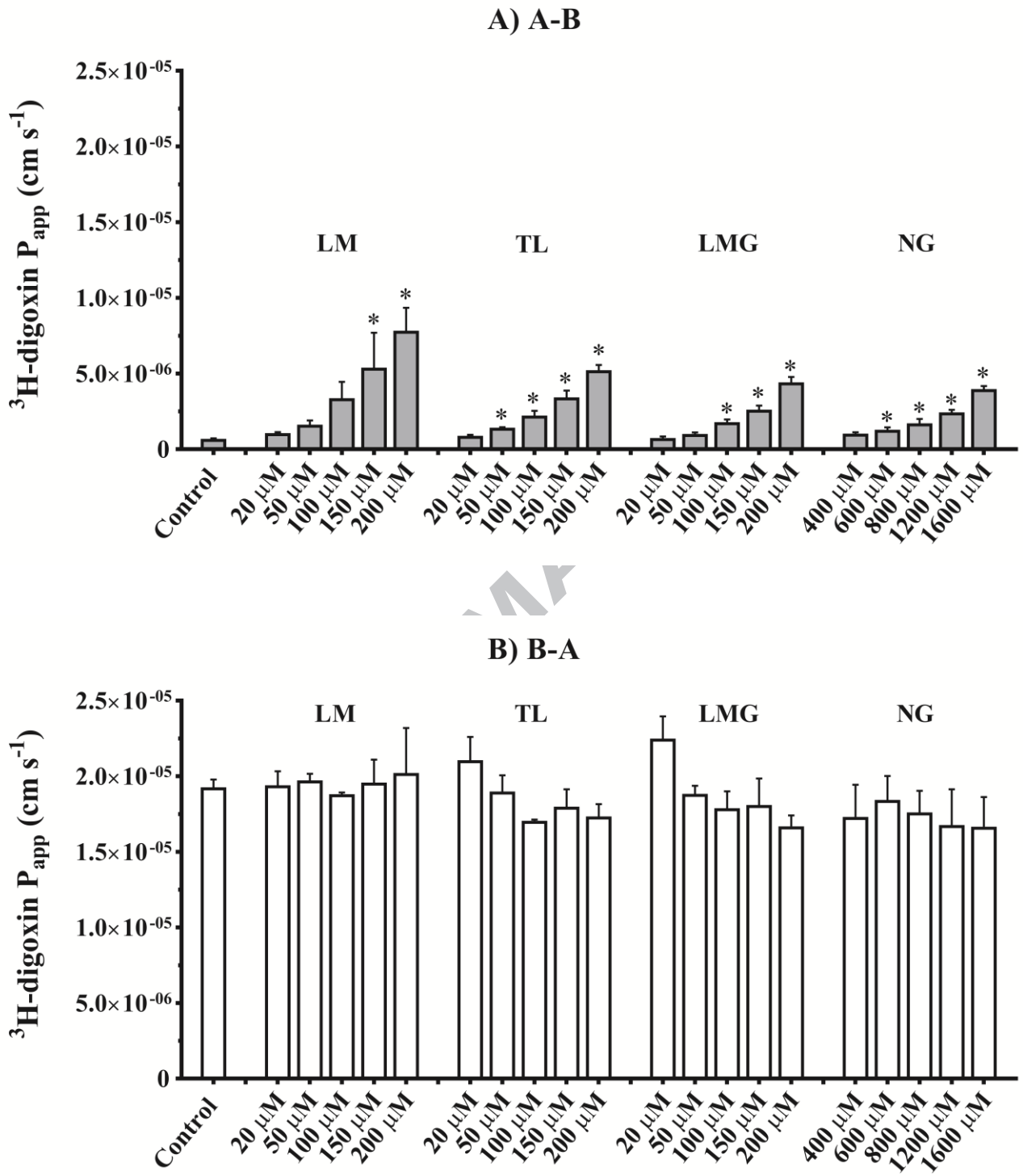


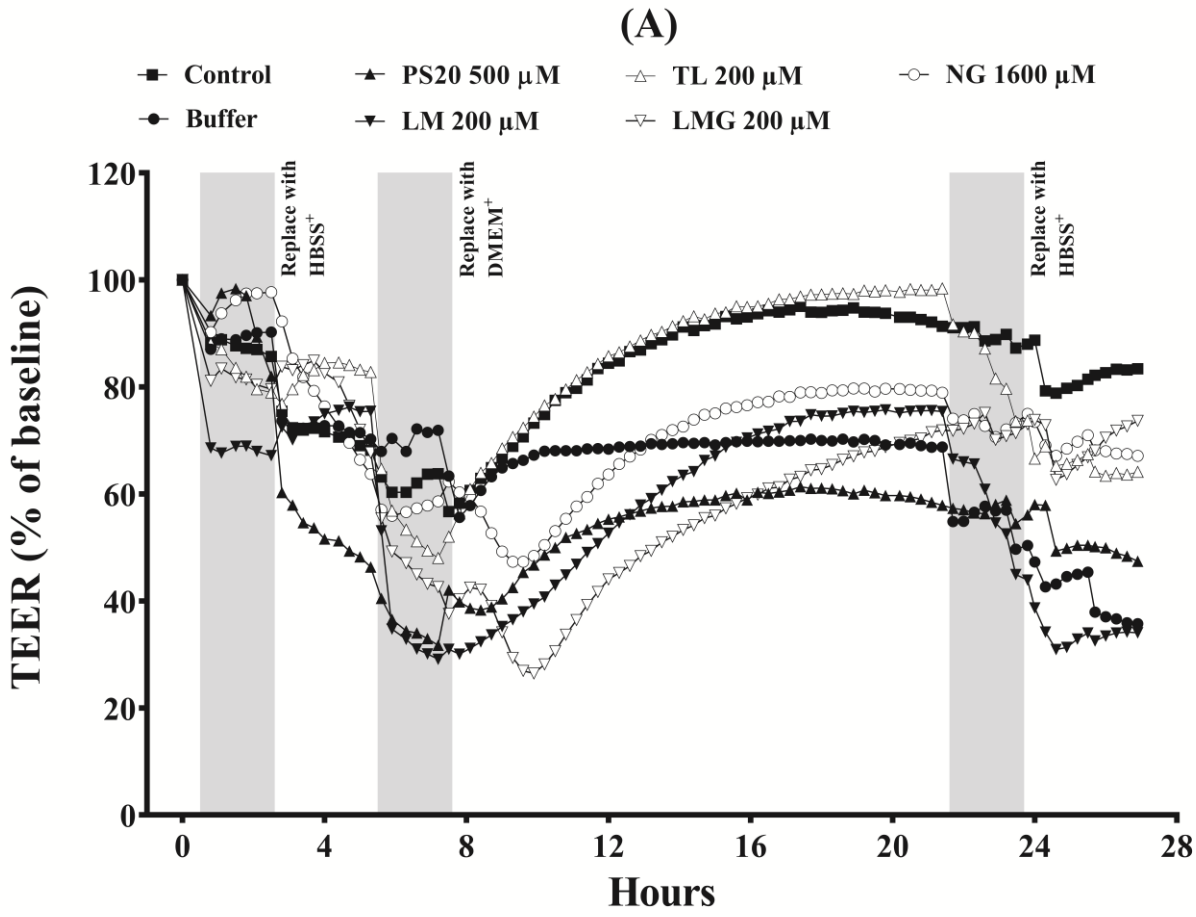
w+x+y+z=20

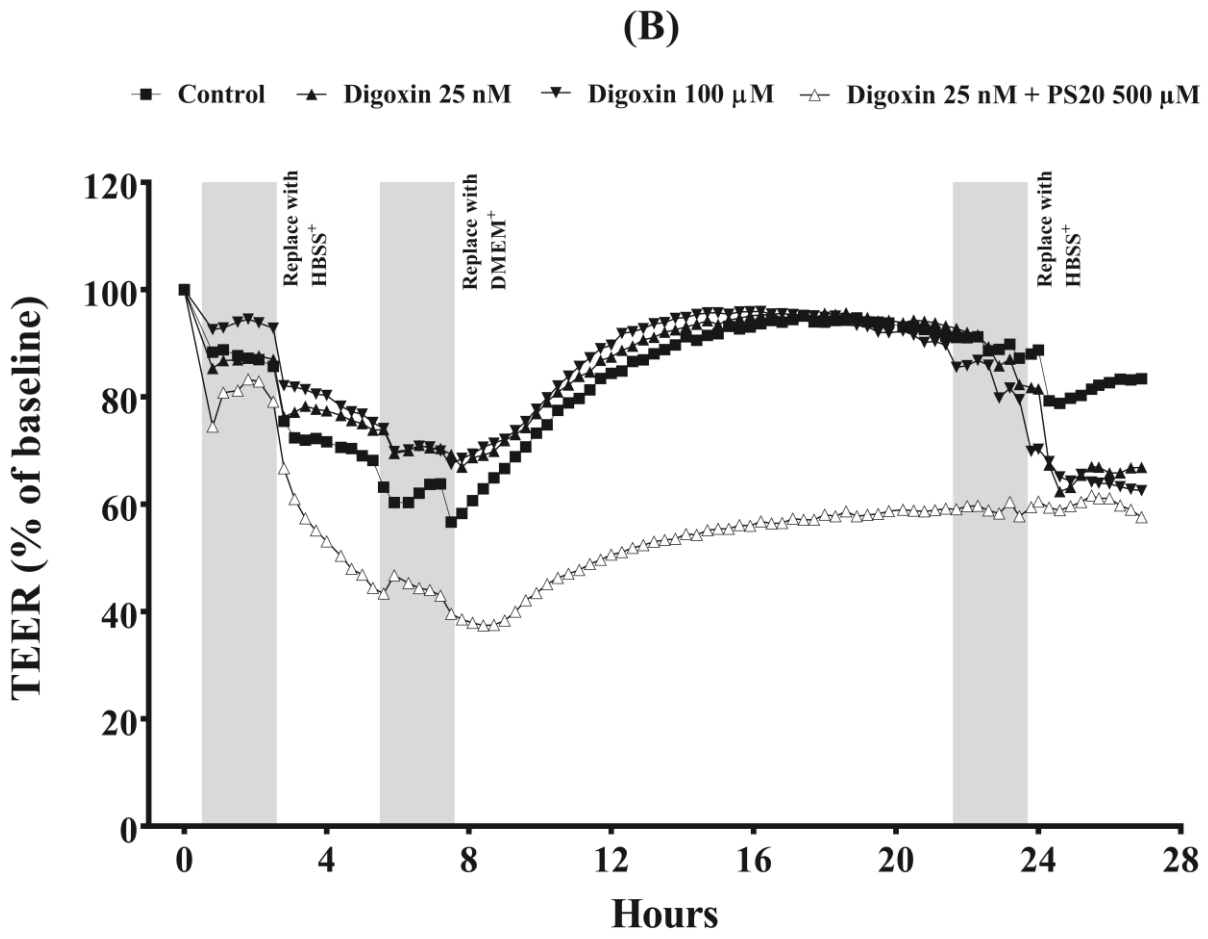
Polysorbate 20: R_1, R_2, R_3 = H; R_4 = -$CO(CH_2)_{10}CH_3$
Polysorbate 40: R_1, R_2, R_3 = H; R_4 = -$CO(CH_2)_{14}CH_3$
Polysorbate 60: R_1, R_2, R_3 = H; R_4 = -$CO(CH_2)_{16}CH_3$
Polysorbate 80: R_1, R_2, R_3 = H; R_4 = -$CO(CH_2)_7CH{=}CH{-}(CH_2)_7CH_3$
Polysorbate 65: R_3= H; R_1, R_2, R_4 = -$CO(CH_2)_{16}CH_3$
Polysorbate 85: R_3= H; R_1, R_2, R_4 = -$CO(CH_2)_7CH{=}CH{-}(CH_2)_7CH_3$


A) A-B
^{3}H-digoxin P_{app} (cm sec^{-1})
2.5×10^{-05}
2.0×10^{-05}
1.5×10^{-05}
1.0×10^{-05}
5.0×10^{-06}
0
*
*
Control
Zosuquidar
PS20
PS60
PS65
PS80
PS85


B) B-A
^{3}H-digoxin P_{app} (cm sec^{-1})
2.5×10^{-05}
2.0×10^{-05}
1.5×10^{-05}
1.0×10^{-05}
5.0×10^{-06}
0
*
*
*
*
*
Control
Zosuquidar
PS20
PS60
PS65
PS80
PS85


A) A-B
B) B-A
^{3}H-digoxin P_{app} (cm s^{-1})
2.5×10^{-05}
2.0×10^{-05}
1.5×10^{-05}
1.0×10^{-05}
5.0×10^{-06}
0
LM
TL
LMG
NG
Control
20 μM
50 μM
100 μM
150 μM
200 μM
400 μM
600 μM
800 μM
1200 μM
1600 μM


(A)
Control
PS20 500 µM
TL 200 µM
NG 1600 µM
Buffer
LM 200 µM
LMG 200 µM
Replace with HBSS+
Replace with DMEM+
Replace with HBSS+
TEER (% of baseline)
Hours
0
20
40
60
80
100
120
0
4
8
12
16
20
24
28



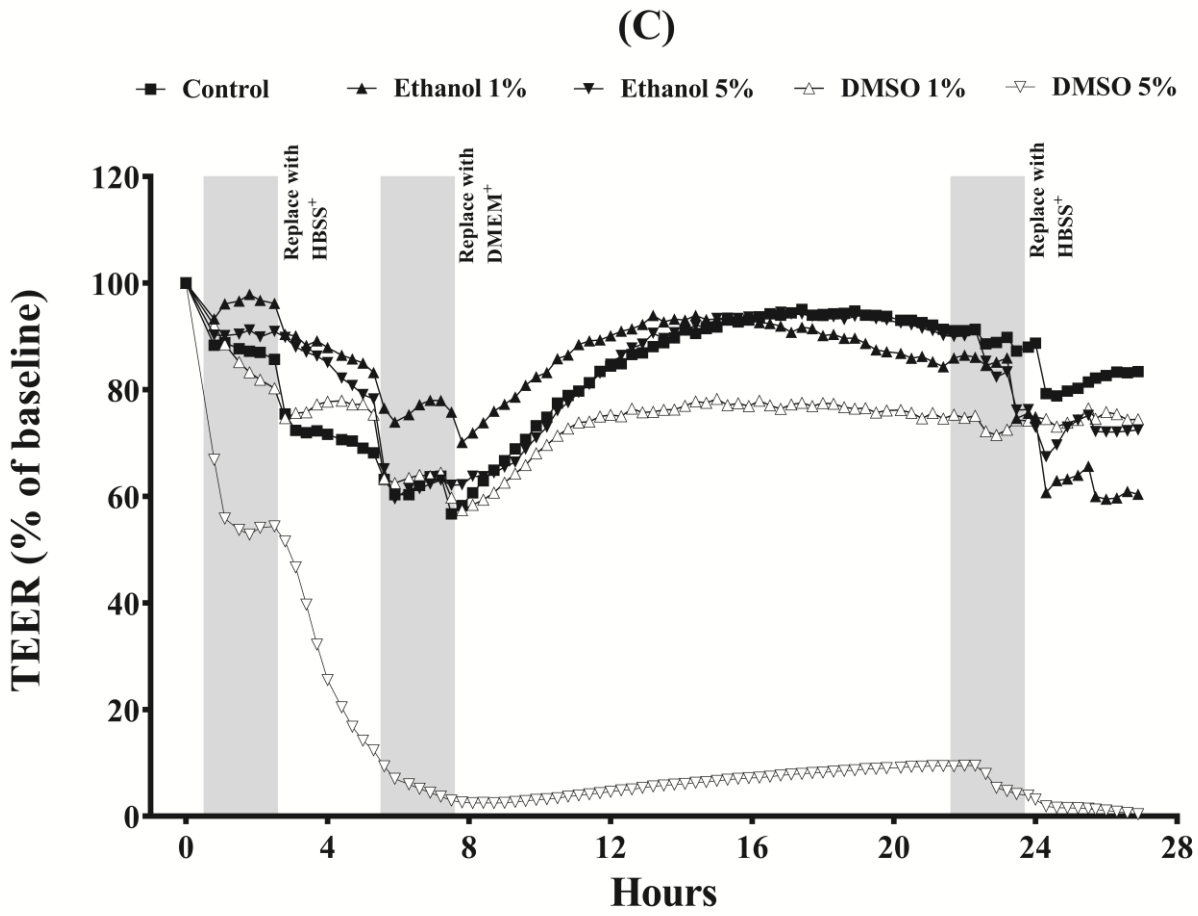

(C)
Control
Ethanol 1%
Ethanol 5%
DMSO 1%
DMSO 5%
Replace with HBSS+
Replace with DMEM+
Replace with HBSS+
TEER (% of baseline)
120
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Hours

Table 1: Specifications of nonionic surfactants

Surfactant	Abbreviation	Molecular weight g mol^{-1} [a]	CMC (µM)	HLB
Polysorbate 20	PS20	1227	49[b], 50[c]	16.7[h]
Polysorbate 40	PS40	1285	24[b], 33[c]	15.6[h]
Polysorbate 60	PS60	1312	21[b], 17[c]	14.9[h]
Polysorbate 65	PS65	1845	22[b]	10.5[h]
Polysorbate 80	PS80	1310	11[b], 15[c]	15.0[h]
Polysorbate 85	PS85	1839	13[b]	11.0[h]
Lauryl-β-D-maltoside	LM	511	180[d], 200[e]	14.5[i]
Trehalose 6-laurate	TL	525	150[a]	14.0[j]
Lauroyl methyl glucamide	LMG	378	347[f]	10.5[k]
n-Nonyl β-D-glucopyranoside	NG	306	6500[g]	11.8[i]

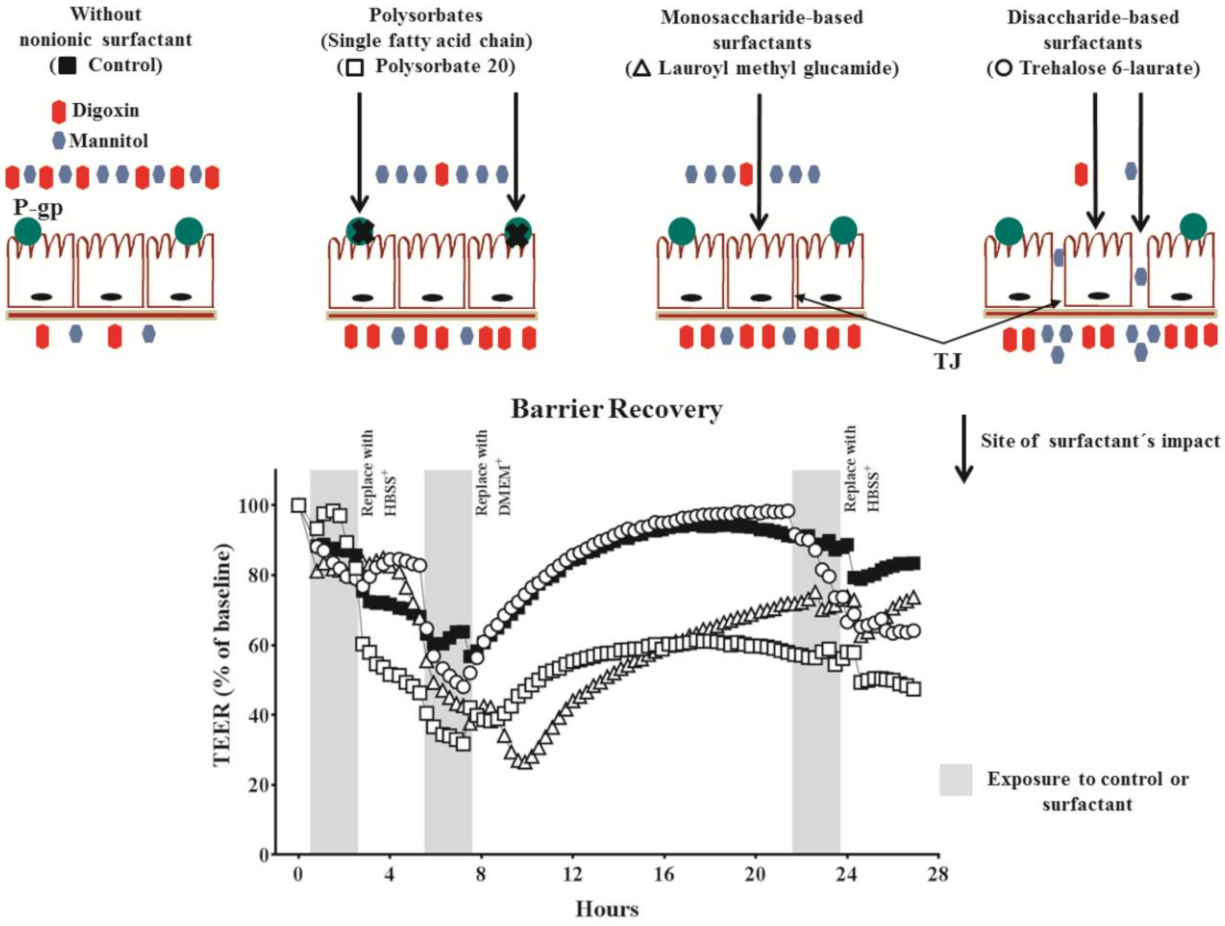
[a]: As described by the manufacturer, [b]: (Wan and Lee, 1974), [c]:(Mahmood and Al-Koofee, 2013), [d]: (Dupuy et al., 1997), [e]: (Zhang et al., 2004), [f]: (Zhu et al., 1999), [g]: (Ericsson et al., 2004), [h]: (Given and Ringleib, 2013), [i]: (Berger et al., 2005), [j]: (Schiefelbein et al., 2010), [k]: Calculated by Griffin's method.

Table 2: Estimated IC_{50} values and 95% confidence intervals (Ci) from calcein-AM efflux experiments in MDCKII MDR1 cells. Data are from 4-17 independent cell passages with 1-3 replicates per experiment. (nd, not possible to determine).

	IC_{50} (µM)	95% Ci of IC_{50} (µM)
Zosuquidar	0.1	0.06 – 0.30
Valspodar	0.8	0.4-1.7
Verapamil	1.8	1.2-2.8
Quinidine	3.8	3.1-4.7
PS20	11	8-15
PS40	60	28-139
PS60	59	34-108
PS65	923	363-1212
PS80	69	34-150
PS85	nd	nd
LM	42	28-62
TL	73	47-114
LMG	88	67-112
NG	551	426-721

Table 3: Absorptive and secretory apparent permeability coefficients (P_{app}) of ^{14}C-mannitol, transepithelial electrical resistance (TEER) and recovery (%) of ^{3}H-digoxin after experiments in Caco-2 and MDCKII MDR1 cells.

	Absorptive				Secretory			
	P_{app} (x 10^{-6} cm s^{-1}) ^{14}C-mannitol	TEER (Ω cm^2)		Recovery (%) ^{3}H-digoxin	P_{app} (x 10^{-6} cm s^{-1}) ^{14}C-mannitol	TEER (Ω cm^2)		^{3}H
µM		Before	After			Before	After	
				MDCKII MDR1				
0	1.37 ± 0.10	136 ± 15	160 ± 25	96 ± 1	0.99 ± 0.12	147 ± 17	153 ± 16	97
10	1.62 ± 0.18			93 ± 1	1.31 ± 0.22			92
200	1.53 ± 0.38	143 ± 18	161 ± 18	113 ± 10	1.12 ± 0.20	153 ± 22	163 ± 31	10
200	1.57 ± 0.29	141 ± 20	166 ± 23	101 ± 5	1.52 ± 0.15	148 ± 25	168 ± 14	10
200	1.30 ± 0.25	145 ± 23	162 ± 20	100 ± 4	1.43± 0.17	156 ± 26	172 ± 16	93
200	1.43 ± 0.37	142 ± 20	171 ± 27	100 ± 3	1.27 ± 0.09	143 ± 19	172 ± 24	10
200	1.15 ± 0.14	143 ± 21	178 ± 31	94 ± 1	1.11 ± 0.12	141 ± 18	174 ± 27	10
				Caco-2				
0	0.50 ± 0.09	516 ± 24	334 ± 12	93 ± 1	0.82 ± 0.11	515 ± 25	311 ± 13	85
20	0.60 ± 0.35	501 ± 54	359 ± 24	90 ± 2	1.08 ± 0.24	557 ± 53	349 ± 36	89
50	0.77 ± 0.43	558 ± 78	370 ± 23	92 ± 1	1.06 ± 0.25	566 ± 93	333 ± 29	94
100	1.23 ± 0.33	574 ± 80	268 ± 15	93 ± 1	1.49 ± 0.38	556 ± 68	232 ± 15	90
150	1.41 ± 0.08	535 ± 80	205 ± 17 *	99 ± 1	2.79 ± 0.93 *	531 ± 105	112 ± 14 *	94
200	3.04 ± 0.45 *	512 ± 53	121 ± 18 *	98 ± 1	3.24 ± 0.75 *	516 ± 51	96 ± 14 *	91
20	0.46 ± 0.09	577 ± 60	321 ± 42	90 ± 2	1.13 ± 0.37	621 ± 57	251 ± 37	90
50	0.45 ± 0.06	614 ± 56	382 ± 18	94 ± 3	1.09 ± 0.18	598 ± 53	300 ± 26	89
100	0.93 ± 0.21	594 ± 60	262 ± 13	96 ± 3	1.15 ± 0.27	588 ± 48	246 ± 73	84
150	1.38 ± 0.29 *	606 ± 67	221 ± 24 *	98 ± 1	1.73 ± 0.28 *	580 ± 50	180 ± 16 *	80
200	1.98 ± 0.30 *	593 ± 48	167 ± 19 *	95 ± 2	2.72 ± 0.39 *	621 ± 46	122 ± 20 *	80
20	0.36 ± 0.02	473 ± 43	345 ± 11	90 ± 1	0.83 ± 0.03	463 ± 36	349 ± 4	10
50	0.36 ± 0.03	467 ± 49	339 ± 12	89 ± 2	0.69 ± 0.07	484 ± 20	326 ± 19	95
100	0.47 ± 0.00	469 ± 37	285 ± 20	91 ± 1	0.89 ± 0.09	465 ± 37	255 ± 11	94
150	0.69 ± 0.06	454 ± 42	210 ± 40 *	90 ± 1	1.27 ± 0.08	421 ± 61	131 ± 29 *	93
200	0.93 ± 0.02	471 ± 36	134 ± 14 *	92 ± 2	1.42 ± 0.13 *	467 ± 34	85 ± 7 *	90
400	0.35 ± 0.02	471 ± 25	343 ± 23	90 ± 4	0.73 ± 0.09	451 ± 24	277 ± 53	88
600	0.38 ± 0.04	475 ± 31	331 ± 28	96 ± 1	0.82 ± 0.09	436 ± 42	290 ± 47	89
800	0.47 ± 0.04	422 ± 32	291 ± 21	99 ± 2	0.80 ± 0.09	443 ± 31	285 ± 18	92
1200	0.48 ± 0.01	489 ± 28	278 ± 19	98 ± 1	0.95 ± 0.07	409 ± 62	246 ± 31	89
1600	0.66 ± 0.07	462 ± 28	211 ± 25 *	99 ± 2	0.90 ± 0.07	388 ± 61	231 ± 27	94


Without
nonionic surfactant
(■ Control)
Digoxin
Mannitol
P-gp
Polysorbates
(Single fatty acid chain)
(□ Polysorbate 20)
Monosaccharide-based
surfactants
(△ Lauroyl methyl glucamide)
Disaccharide-based
surfactants
(○ Trehalose 6-laurate)
TJ
Barrier Recovery
Site of surfactant´s impact
Replace with HBSS+
Replace with DMEM+
Replace with HBSS+
TEER (% of baseline)
Hours
Exposure to control or surfactant